Arginase, Prostaglandins, and Myeloid-Derived Suppressor Cells in Renal Cell Carcinoma

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Abstract

Tumor-induced tolerance is a well-established phenomenon in cancer patients that can severely impair the therapeutic efficacy of immunotherapy. One mechanism leading to T-cell tolerance is the generation of myeloid-derived suppressor cells (MDSC) by soluble factors produced by the tumor. MDSC express CD11b+ as a common marker but may vary in their stage of maturation, depending on the tumor factors being produced. Arginase production by MDSC depletes arginine from the tumor microenvironment and impairs T-cell signal transduction and function. We studied whether an increase in MDSC could explain the molecular alterations and dysfunction found in T cells of patients with renal cell carcinoma (RCC). Arginase activity in the peripheral blood mononuclear cells of 117 RCC patients was increased between 6- to 8-fold compared with normal controls. The increased arginase activity was limited to the CD11b+CD14+ myeloid cells and resulted in significantly decreased serum levels of arginine and increased ornithine in patients. Depletion of MDSC restored IFN-γ production and T-cell proliferation. Preliminary data suggest that prostaglandin E2 produced by the tumor induces arginase I expression in MDSC. Therefore, blocking MDSC activity may enhance the therapeutic efficacy of immunotherapy in RCC.

Immunotherapy with cytokines, such as interleukin-2 (IL-2), has become a standard of care for patients with renal cell carcinoma (RCC). However, only 20% to 30% of patients have a partial or complete response (1), which is significantly lower than the therapeutic efficacy suggested by animal models. One possible explanation is that tumor-induced tolerance diminishes the potential therapeutic effect of T cells, the principal effector cells in most forms of immunotherapy. Over the last decade, several mechanisms by which tumors escape the immune response have been described. These can be divided into three major groups: alterations in antigen expression in tumor cells to make them less detectable, the active suppression of dendritic and T-cell function by inhibitory molecules or factors produced by tumors, and the induction of cells that can suppress the immune response, including myeloid-derived suppressor cells (MDSC) and regulatory T cells. We have focused our work on characterizing MDSC and the mechanisms by which they cause T-cell dysfunction in cancer.

MDSC in Murine Tumor Models

MDSC comprise a phenotypically heterogeneous population of cells found in mice with tumors and patients with cancer that very efficiently suppress T-cell function. These cells are induced from bone marrow progenitors and vary in their maturation markers, depending on the combination of factors produced by the tumor, including vascular endothelial growth factor, granulocyte macrophage colony-stimulating factor, and macrophage colony-stimulating factor (2). As such, MDSC found in mice bearing s.c. implants of colon carcinoma CT26 have been described as “myeloid suppressor cells” that are CD11b+GR1+ that produce nitric oxide and arginase I (2, 3). In contrast, MDSC infiltrating 3LL lung carcinoma express mature macrophage markers CD11b+GR1+CD68+ (4, 5) and express high levels of arginase I (Fig. 1A). Light microscopy and immunostaining confirm the morphology of these cells to be macrophages with a high expression of arginase I (Fig. 2A and B). The coculture of MDSC with T lymphocytes rapidly depletes arginine from the microenvironment, causing a profound inhibition of T-cell proliferation, cytokine production, and the expression of the CD3ζ chain of the T-cell receptor (Fig. 1B; refs. 5, 6). In certain tumor models, the coexpression of arginase I and nitric oxide synthase allows MDSC not only to suppress T-cell function but also to induce T-cell apoptosis (4). The molecular mechanisms by which MDSC inhibit T-cell function are subject of extensive research. Arginase I is not exported by murine MDSC into the tumor microenvironment or the serum of tumor-bearing mice. Instead, MDSC express the cationic amino acid transporter CAT-2B, which allows them to...
rapidly incorporate arginine, effectively depleting this amino acid from the surrounding microenvironment. Therefore, it is possible that tumor-infiltrating lymphocytes stimulated by tumor antigens in this arginine-depleted microenvironment develop defects, such as the loss of CD3 chain of the T-cell receptor, and become anergic. More recent data show that T cells cultured in arginine-free conditions are arrested in G0 phase of the cell cycle by the inability to up-regulate cyclin D3 and cyclin-dependent kinase 4 upon antigen stimulation.2

Murine immunotherapy models have confirmed the importance of arginase in inhibiting T-cell function. Treatment of tumor-bearing animals with s.c. injections of specific arginase inhibitors, such as nor-hydroxy arginine, results in a dose-dependent inhibition of tumor growth as shown in Fig. 3. This antitumor response is dependent on the presence of a competent immune system because treatment of tumor-bearing severe combined immunodeficient (scid) mice with nor-hydroxy arginine failed to prevent tumor growth (5). Similarly, the use of nitro-aspirin, a compound that primarily inhibits nitric oxide synthase, can also result in an increased T-cell response to vaccination with tumor antigens (5). Both arginase I and nitric oxide synthase use arginine as their main substrate; therefore, inhibition of these enzymes may lead to the replenishment of arginine and the re-establishment of a therapeutic immune response. Unfortunately, simple replenishment of arginine alone by s.c. injection only partially delays the growth of the tumor in mice, suggesting that once T-cell dysfunction is established, its reversal will require additional stimulatory signals, such as antigen and/or cytokines. Alternatively, as shown in some clinical trials, arginine may also provide a growth stimulus to tumor cells (7).

MDSC in Patients with Metastatic RCC

Increases in arginase activity have been previously reported in patients with cancer but were thought to reflect an increased metabolic rate of the tumor cells. The murine data suggested instead that the increased arginase activity was the result of an increased number of metabolically active MDSC. We therefore tested whether an increased arginase activity was seen in RCC patients. Frozen peripheral blood samples from 117 metastatic RCC patients were collected before the initiation of treatment with IL-2 or IL-2 and IFN-α and tested for arginase activity. The results showed a significant (P < 0.001) increase in arginase activity in the peripheral blood mononuclear cells (PBMC) of patients (150.3 ± 114.0; range, 2.5-476.0) compared with normal controls (14.25 ± 11.91; range, 1.8-46.87; Fig. 4A). Patient PBMC also had a decreased z chain expression in T and natural killer cells (mean fluorescence intensity = 21.20 versus 37.90 for controls; Fig. 4B).

Additional studies aimed at identifying the MDSC were then done in PBMC samples from 15 newly diagnosed RCC patients. Flow cytometry analysis showed an increased in a subpopulation of large granular cells that were CD11b+CD14 (Fig. 5A). Additional phenotyping showed that these cells expressed CD15, CD80, CD83, CD86, MHC class II, and CD11a (8). In

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contrast to murine MDSC, Wright staining showed these cells to have polymorphonuclear granulocyte morphology (Fig. 5B). Western blot analysis confirmed the high expression of arginase I in these cells and not in other subpopulations of PBMC (Fig. 5C). Other soluble mediators known to suppress T-cell function, such as indoleamine 2,3-dioxygenase and hydrogen peroxide, were not found in these cells. Not surprisingly, statistical analysis showed a direct correlation between the percentage of CD11b+CD14+ MDSC and arginase activity in PBMC (\(r^2 = 0.9327\)).

Arginase I metabolizes arginine to ornithine. Patients with severe trauma, who also have an increased arginase activity in PBMC, have a rapid decline in serum arginine levels (9, 10). Mean plasma levels of arginine were significantly decreased in the 15 patients studied (28.48 ± 32.51 μmol/L) compared with controls (90.0 ± 15.56 μmol/L) (Fig. 6). Conversely, ornithine levels were increased in patients (172.1 ± 61.01) compared with healthy controls (81.5 ± 26.16). Therefore, MDSC not only expressed arginase I but also produced metabolic effects, reducing arginine and increasing ornithine in serum.

Previous reports have shown that T cells from RCC patients proliferate poorly after stimulation with anti-CD3 and anti-CD28. If MDSC are in part responsible for the impaired T-cell response, their depletion should eliminate this effect. Indeed, in vitro depletion of CD11b+ cells but not CD14+ cells from the peripheral blood samples re-established T-cell proliferation (Fig. 7A), IFN-γ production (Fig. 7B), and re-expression of the T-cell receptor CD3ζ chain (Fig. 7C). Therefore, an increase in MDSC leading to a depletion of arginine in serum may explain why the molecular and functional alterations in T cells are not only limited to tumor-infiltrating lymphocytes but may also affect peripheral T cells.

**Cyclooxygenase-2 and Arginase in MDSC**

It is unclear what factors induce arginase in MDSC in human cancer patients. In vitro models showed that stimulation of peritoneal murine macrophages with cytokines, such as IL-4, IL-13, transforming growth factor-β, and IL-10, induced arginase I expression. However, extensive studies using the murine 3LL lung carcinoma model failed to show the production of any of these cytokines by the tumor cells or in the tumor microenvironment. Instead, 3LL cells produced high levels of prostaglandin E2, which rapidly induced arginase I expression in MDSC-infiltrating tumors. Furthermore, the inhibition of cyclooxygenase-2 in vitro and in vivo blocked arginase expression and induced an antitumor response that was mediated by T cells (11). In humans, understanding the mechanisms that induce arginase has been more complex. Studies by Munder et al. (12) have shown that granulocytes have an increased expression of arginase I. They have also shown that incubation of human monocytes or granulocytes from infected patients with multiple cytokines does not lead to a significant increase of arginase I. Therefore, the mechanism of arginase induction in human tumors is still unclear. We believe that prostaglandin E2 may induce arginase in the human granulocytes, as previously shown in mice (11), especially because cyclooxygenase-2 expression is increased in many malignancies (13, 14).

**Discussion**

Tumor-induced T-cell tolerance is by now a well-established observation in murine tumor models and patients with cancer.
Although the mechanisms are still incompletely understood, there is a consensus that tumor-induced anergy can severely impair the efficacy of the various forms of immunotherapy being tested in clinical trials. Several possible mechanisms inducing tolerance have been proposed; however, these vary between murine models and may be different for each type of tumor. The different mechanisms can be divided into three major categories, including changes in the expression of HLA and tumor-associated antigens in the malignant cells, the induction of regulatory T cells or MDSC (15, 16), and the inhibition of dendritic and T-cell function by tumor-derived factors (17–19).

MDSC have been reported in the spleen of mice with colon, prostate, lung, and breast cancer. They suppress T-cell proliferation through a combination of nitric oxide and arginase production. Arginase depletes arginine from the microenvironment, leading to distinct molecular changes in T cells, including the loss of T-cell receptor signaling and the inhibition of cell cycle in G0 phase. These changes can lead to peripheral T-cell tolerance. In addition, the production of nitric oxide may eventually lead to T-cell apoptosis in the tumor microenvironment where there is cell-cell contact (4, 20, 21). Of these two mechanisms, arginase I production seems to be the primary mechanism by which MDSC induce T-cell tolerance. Blocking arginase activity in vitro or in vivo with specific inhibitors eliminates the suppressor activity and allows the development of an effective antitumor response against s.c. tumor implants. MDSC have clearly been shown in cancer patients. Although the mechanisms for the induction of T-cell dysfunction are similar to those described in mice, the mechanisms inducing arginase in granulocytes are still unclear.

How low does arginine have to be to impair T-cell function in vivo? Normal levels of arginine in human serum range from 50 to 150 μmol/L. Patients with severe trauma have a rapid increase in arginase activity in PBMC and profoundly depleted arginine levels in plasma (between 0 and 50 μmol/L). This is accompanied by T-cell anergy and loss of CD3ζ expression (22, 23). Similarly, RCC patients have a decrease in arginine levels compared with normal controls. This is significant because Taheri et al. (24) and Rodriguez et al. (25) showed in vitro that arginine concentrations below 60 μmol/L decreased CD3ζ expression, cell proliferation, and cytokine production. In addition, MDSC producing arginase have been found infiltrating RCC. Therefore, it is possible that arginase production and arginine depletion in the tumor or lymphoid organ microenvironment may be even more profound than...
that seen in serum. Unfortunately, the simple replenishment of arginine to overcome this mechanism does not seem to be a simple solution to these problems because it could stimulate tumor growth (5, 7, 26).

It is possible that these circulating myeloid cells represent a subset of the arginase-producing myeloid cells infiltrating the tumor. However, they may also represent a subpopulation of immature cells induced by vascular endothelial growth factor (27, 28). Preliminary data suggest that the morphology is similar to activated granulocytes described by Schmielau and Finn (19). Clinical trials have also suggested that granulocytes may have a negative effect on the outcome of patients receiving immunotherapy. Donskov et al. (25) studying RCC patients receiving IL-2–based therapies, showed that an increased number of neutrophils in peripheral blood of RCC patients was associated with poor clinical response and outcome. However, these studies did not measure the levels of arginase as a correlate to neutrophil numbers or outcome.

Regulation of specific amino acids is a novel and important mechanism for tumor escape. In addition to arginine, the depletion of tryptophan by cells expressing indoleamine 2,3-dioxygenase described by Heys et al. (26) also inhibits T-cell proliferation and sensitizes T cells to apoptosis. Understanding the molecular mechanisms may provide insights into novel therapeutic approaches to block the suppressor mechanisms and enhance the therapeutic effect of immunotherapy.

**Open Discussion**

**Dr. Kwon:** Do you have data pertaining to other malignancies as well?

**Dr. Ochoa:** Yes, we have studied patients with head and neck, lung, and colon carcinoma. In all of these, you see a similar population of myeloid suppressor cells that are producing arginase 1. I believe that different tumors will induce myeloid cells at different stages of differentiation, depending on the type of tumor-derived soluble factors, such as vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor, etc. In general, however, we have decided to call them myeloid-derived suppressor cells. If we can block their induction or prevent their function, our ability to induce an antitumor effect with vaccines or with interleukin 2 (IL-2) is probably going to increase.

**Dr. Sosman:** A previous study has looked at immature myeloid cells and found that the mobilization to the tumor was VEGF dependent, and they secreted matrix metalloproteinase 9, which is a key factor in angiogenesis. They even saw that some of these myeloid cells appear to be incorporated.

**Dr. Ochoa:** Although we have generalized these cells as immature myeloid cells, they are not necessarily immature. Some tumor models, for example, the 3LL lung carcinoma, induce mature macrophages that produce arginase and have all the maturation markers. The stage of maturation will probably depend on the factors secreted by the tumor.

**Dr. Kwon:** Renal cell carcinoma is one malignancy where the extent of inflammation, paradoxically, portends to poor outcome. Therefore, the more mononuclear cell infiltration you have, the poorer your prognosis. What is lacking from the literature is a detailed analysis of what that mononuclear cell infiltrate is composed of.

**Dr. Ochoa:** It is also interesting that when one separates peripheral blood mononuclear cells from these patients with regular Ficoll-Hypaque, these granulocytes producing arginase are found in the mononuclear band of cells, suggesting these granulocytes are activated as in an inflammatory process.

**Dr. Ernstoff:** There are also data on tumor-infiltrating dendritic cells. We have shown that in renal tumors, these DCs are dysfunctional. It is potentially that this tumor infiltrate is actually a potent down-regulator.

**Fig. 7.** Indeed, in vitro depletion of CD11b+ cells but not CD14+ cells from the peripheral blood samples re-established (A) T-cell proliferation, (B) IFN-γ production, and (C) re-expression of the T-cell receptor CD3ζ chain.

Dr. Figlin: Does the fact that these tumors are known to secrete IL-6 help explain that phenomenon?

Dr. Kwon: I have no idea.

Dr. Vieweg: Every tumor creates its own microenvironment influenced by tumor-derived factors. We have to be careful in recommending therapy because we have to analyze the specific immunosuppressive profile from every patient.

Dr. Ochoa: Now that you are talking about therapy, one of the other things that we need to be careful of is that you can’t just give arginine to these patients because some tumors, breast cancer and prostate cancer specifically, make their own arginase.

Dr. Atkins: This discussion at least raises the potential that binding or blocking VEGF might be immunomodulatory, and there might be some way in which one could synergistically combine agents such as bevacizumab and IL-2.

References


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